Bioremediation techniques for naproxen and carbamazepine elimination. Toxicity evaluation test

KEYWORDS: Naproxen, carbamazepine, wastewater treatment stations, toxicity.

Abstract Isolation, identification and toxicological monitoring of new microbial consortia to degrade PPCP’s (pharmaceuticals and personal care products) have direct applications to improve the quality of effluents in WWTP (wastewater treatment plants). In this work, we present isolation and molecular identification of a microbial strain degrading frequently detected compounds in effluent water such as naproxen (analgesic) and carbamazepine (anticonvulsant), as well as detoxification evaluation by a zebrafish embryos biomodel. Naproxen was completely removed from real contaminated water samples within 7 days of culture by Serratia sp. However, the microbial strain was unable to remove carbamazepine remaining constant until the analysis finished. Toxicity test showed that naproxen elimination reduces mortality zebrafish embryos at 28%. As conclusion, Serratia sp. and possible other Enterobacteriaceae could be used in bioreactors and purifying plant biodiscs in order to achieve naproxen and probably others PPCPs total elimination from effluent prior being poured into rivers or lakes.

INTRODUCTION

PPCP’s (pharmaceuticals and personal care products) are a large group of chemicals covering all pharmaceuticals for both human and animal use, diagnostic agents, vitamin supplements and other fine chemicals such as fragrances or sunscreens. In the 90s they were known as emerging water contaminants (1) due to the potential impact in different environmental compartments (2). Most of PPCP’s found in wastewater treatment plants are hormones (30%), analgesics and anti-inflammatory (20%) and antibiotics (9%) (3). Ibuprofen, diclofenac or carbamazepine are some examples of chemicals found in drinking water (4). A plan ensuring the quality of water is required for all the competent authorities of each member state at 2015 by Water Framework Directive (2000/60/EC). Nevertheless, a maximum limit of these compounds present in drinking water has not been setting yet by the European Union. Therefore, PPCP’s are of great interest both at scientific and social levels. Urban wastewater is one of the polluting environment paths due to an assimilation lack and subsequent excretion by human body, elimination of chemicals down the drain or by urban solid waste followed by leaching into the aquatic environment. A higher pharmaceuticals concentration is present in wastewater from both hospitals and PPCP’s manufacturing companies (5). Moreover, gradual aging of population particularly marked in Spain suggests a growing consumption of drugs in the next years. In fact, Spain is seventh placed by the World Health Institute related to world consumption of drugs. Finally, livestock is also considered an important way of ground and surface water pollution because of chemicals consumption from animals and subsequent contamination of manure used as fertilizer. Many efforts are being made by the scientist community to determine the presence and risk of these pollutants in aquatic systems trying to remove them by wastewater treatments and recycling processes. Current pollutants concentrations are usually in a range between ng L⁻¹ and mg L⁻¹ (6). Even lower concentration levels of PPCP in waters affect human health due to their potential biomagnification through the food chain in aquatic organisms (7). The use of membrane bioreactors (MBR), ozonation processes and advanced oxidation (AOP) (6), activated carbon adsorption (8), photocatalytic treatments (9) or ultraviolet photodegradation (10) are novel techniques for PPCP’s removal. However, the microorganisms efficiency (isolates or consortia) for degrading contaminants is presented as an alternative to those techniques. In this case, the complete pollutants transformation into nontoxic substances (CO₂, N₂, H₂O) as well as low economic,
The objectives of the present study are:

1. Isolation and molecular identification of degrading bacteria
   - Three cultures were prepared from each sample (final volume 50 mL). 1 mL of sample was inoculated into BHB (Bushnell Haas Broth) culture medium containing Tween-80 (1%) as surfactant and carbamazepine (0.25 ppm) or naproxen (1.25 ppm) as pollutant. Tween-80 is a surfactant, which enhances the solubility of PPCPs. A liquid culture medium in BHB with Tween-80 (1%) was prepared from each colony biomass using carbamazepine (0.125 ppm) or naproxen (0.625 ppm) as carbon sources. Optical density evolution of 27 isolated colonies from water samples over 12 days was studied. Liquid cultures that showed cell growth (increment of the cell density), were refreshed with liquid media to avoid that nutrients were consumed. The isolates were cultivated again in a liquid media with PPCPs as sole carbon source and followed by a new track over 19 days under the same conditions mentioned above.

2. Detoxification process evaluation by a biomodel with zebrafish embryos
   - In order to obtain biomass all that isolated colonies were replated under the same conditions in individual plates. A liquid culture medium in BHB with Tween-80 (1%) was prepared from each colony biomass using carbamazepine (0.125 ppm) or naproxen (0.625 ppm) as carbon sources. Optical density evolution of 27 isolated colonies from water samples over 12 days was studied. Liquid cultures that showed cell growth (increment of the cell density), were refreshed with liquid media to avoid that nutrients were consumed. The isolates were cultivated again in a liquid media with PPCPs as sole carbon source and followed by a new track over 19 days under the same conditions mentioned above.

Naproxen and carbamazepine degradation kinetics
   - 1 mL of a dilution in which was calculated the most probable number (MPN) was incubated in a final volume of 30 mL with a carbamazepine and naproxen

EXPERIMENTAL

Sewage water collection
Water samples were collected from urban WWTP. The main supply is wastewater from two populations included in Community of Madrid. This plant pours water to a stream from the secondary treatment and purifies also through a tertiary treatment to the required quality for use as irrigation water. We collected three types of samples: riverbed water, sample after secondary treatment (just before pouring the stream) and water after completing tertiary treatment (irrigation water for agricultural use). Samples were refrigerated at 4 ºC until arrival at the laboratory.

Isolation and molecular identification of degrading bacteria
Three cultures were prepared from each sample (final volume 50 mL). 1 mL of sample was inoculated into BHB (Bushnell Haas Broth) culture medium containing Tween-80 (1%) as surfactant and carbamazepine (0.25 ppm) or naproxen (1.25 ppm) as pollutant. Tween-80 is a surfactant, which enhances the solubility of PPCPs. A liquid culture medium in BHB with Tween-80 (1%) was prepared from each colony biomass using carbamazepine (0.125 ppm) or naproxen (0.625 ppm) as carbon sources. Optical density evolution of 27 isolated colonies from water samples over 12 days was studied. Liquid cultures that showed cell growth (increment of the cell density), were refreshed with liquid media to avoid that nutrients were consumed. The isolates were cultivated again in a liquid media with PPCPs as sole carbon source and followed by a new track over 19 days under the same conditions mentioned above.

All incubations were performed on an orbital shaker (New Brunswick Scientific, New Jersey USA) at 200 rpm and 25ºC in darkness to avoid possible influence of light on chemicals stability and contamination by other microorganisms. The colony that showed the best growth (C10) was obtained from the effluent treatment plant discharge directly to the stream (after receiving secondary treatment).

Finally, strain C10 was identified by extracting DNA from the colony C10 and subsequent amplification and sequencing as described in Molina et al., 2009 (22).
A toxicological test with a model zebrafish embryo-larval risk assessment approach was performed using a concentration of 0.75 and 0.45 ppm, respectively. Cell growth was monitored by optical density at 600 nm and the pH of the medium (data not shown). In this case, triplicate samples of each culture were taken over time until a total of 21 days. Samples were centrifuged at 13,000 rpm and 21°C for 10 min, in order to remove cellular material. The supernatant was used as a target and the estimated value was corrected from an aliquot of the same volume without centrifugation. An extraction method using chloroform was developed to assess PPCP’s content in the samples. The incubation medium (30 mL) was collected and naproxen and carbamazepine were extracted in a separatory funnel with 3x5 mL of chloroform. The 3 organic phases were collected, pooled, evaporated and resuspended in 2 mL of acetonitrile (ACN) and then filtered through a 0.2 μm cellulose membrane. Degradation process was analyzed by high performance liquid chromatography (HPLC) on a reverse phase liquid chromatograph equipped with a Shimadzu UV-visible detector and Phenomenex® Luna® C18 (2) (7.5 cm x 4.6 mm, 3μm) column. Analyses were performed in isocratic mode, using a mobile phase acetonitrile:acid water (pH 4.0) 40:60 (v/v). The injection volume was 10 μL at a flow rate of 0.5 mL/min. Both naproxen and carbamazepine quantification was performed measuring characteristic peaks areas of each compound using the corresponding calibration standards (carbamazepine and naproxen analytical standards, ≥ 99.0% HPLC, supplied by Sigma-Aldrich).

Toxicological evaluation

A toxicological test with a model zebrafish embryo-larval (Brachydanio rerio) was used to assess the toxicity of the resulting samples and subsequently the treatment efficiency. Water samples were collected at different stages: an initial one before PPCP’s degradation by microbial strain selected, 1-2 intermediate stages and final stage of pollutants degradation. All water samples were filtered to remove microbial fraction before exhibition to zebrafish embryos. Water samples toxicity with zebrafish embryo-larval model was assessed by mortality (every 24 h of development), malformations (24 h, 48 h and 72 h of development), sublethal alterations such as cardiotoxicity (development of 48 h), growth (72 h of development) and behavior (development of 144 h).

Modelling the PPCPs metabolic kinetics

One of our goals is to model the kinetics of the bacteria-drugs reactions, so we will be able to predict the outcome of future experiments. In order to extract the reaction rates from the experimental data, we have used the method suggested in (30) to optimise the Picard integral operator associated to first order differential equations. Our very preliminary results are plotted in Figure 2 as solid lines. They are in good agreement with the experimental data (also plotted in the same Figure 2, with their corresponding error bars), revealing the behaviour of the concentrations and bacteria population that we discuss in the following section.

RESULTS AND DISCUSSION

Naproxen was completely removed by C10 strain molecularly identified as Serratia sp. within 7 days of culture. However, carbamazepine concentration remained constant until the end of analysis (Figure 2).

To our knowledge this was the first time that a strain of Serratia is able to degrade naproxen. Other works described the PPCP’s degradation (ibuprofen and naproxen) by Pseudomonas putida (23). Moreover, both river native communities (17) and bacteria from air sludge reactors (24) were able to easily degrade naproxen as well. The Gram-positive strain Planococcus sp. removed approximately 30% of naproxen after 35 days of incubation in monosubstrate culture. Under cometabolic conditions, with glucose or phenol as a growth substrate, the degradation efficiency increased (25). In our experimental design where Tween-80 is added as surfactant, naproxen degradation is favoured because this surfactant is also used as carbon source (11). Molecules with more complex structures such as carbamazepine are more difficult to remove with bioremediation techniques (26). However, Rodríguez-Rodríguez et al. (27) and Marco Urrea et al. (28) published that Trameces versicolor degraded both naproxen and carbamazepine (50-90%).

It is known that bioassay models are sensitive to the presence of naproxen. Even at low concentrations if mortality is not caused at least disturbance mobility is
induced [29]. In our experiments, although naproxen was completely removed, zebrafish embryos showed toxicity when they were treated with degradation media for 1, 7 and 21 days. These results are in agreement with carbamazepine presence and probably with toxic intermediates derived from naproxen degradation. However, when sample corresponding to 21 days was filtered removing the microbiological component of the media tested, mortality was reduced by 28%, indicating that the total elimination of naproxen partially reduces the toxicity of the filtrate (Table 1).

Moreover, while blank tests I and II showed low mortality, significant physiological changes such as heartbeat reduced, and circulatory stasis and chorion deterioration caused (Figure 3). Therefore, in absence of PPCPs, both Tween-80 (a detergent that improves the contaminants solubility and also modifies biological membranes) and nutrients (BHB) possibly change osmotic potential of the medium and subsequently disturb biological systems.

CONCLUSIONS

The strain isolated from urban waste water treatment plant has been molecularly identified as Serratia sp. This Enterobacteriaceae has the metabolic capacity to degrade naproxen efficiently but not carbamazepine. Naproxen elimination reduced zebrafish embryos mortality at 28%. The probability to find other Enterobacteriaceae with the capacity to degrade PPCPs is very high according to the natural environment where those microorganisms are found. Therefore, this strain is a potential microorganism for being used in bioreactors and sewage biodiscs in order to achieve the total elimination of naproxen from effluent before discharge into rivers or lakes.

Table 1. Mortality results and physiological effects on zebrafish embryos over time in experiments with and without chemicals. Degraded medium after 21 days was also filtered to remove the possible negative effects of the microorganisms presence before contact with embryos.